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In this project, we are using a novel intrabody approach which we have recently developed to phenotypically knock-out cyclin E in order to determine the critical role of cyclin E in establishing and maintaining the transformed phenotype of breast cancer cells, and to demonstrate whether cyclin E can be used as a therapeutic target. In last 10 months, the variable region cDNA genes of two anti-cyclin E antibodies against the cyclin box domain have been cloned, and sequenced. The cDNA genes were assembled into single-chain antibodies (sFv) consisting of immunoglobulin heavy- and light-china variable domains joined by a flexible peptide linker. The sFv fragments were then cloned into various expression vectors. We are in the process to characterize the binding activities of anti-cyclin E sFvs by using a novel recombinant phage antibody system. We will generate breast cancer cell lines expressing the anti-cyclin E intrabodies to examine the ability of the anti-cyclin E intrabodies to bind cyclin E in vivo and their effects on the the morphology, growth properties and cell cycle progression of untransformed human fibroblasts and breast cancer cells.

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Jchen 6/23

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Introduction

Cyclin E is pivotal in the coordinated regulation of the cell cycle by activating the cyclin-dependent kinase (CDK)-2, suggesting that deregulated expression of cyclin E may cause loss of cell cycle control and thus enhance oncogenesis. Cyclin E has been found to be overexpressed and remain in an active complex with CDK2 throughout the cell cycle in almost all of breast cancer cell lines and patient samples examined so far. In spite of the apparent significance of the altered cyclin E expression, the role of the cyclin E overexpression in transformation and proliferation of breast cancer cells has not been established. Whether blockade of the cyclin E expression can lead to revert transformed phenotype and thus provide a therapeutic target is also unknown. In this application, we propose to use a novel intrabody approach which we have recently developed to phenotypically knock-out cyclin E. **The hypothesis of this proposed study** is that anti-cyclin E intrabodies targeted at the cytosol or nucleus of breast cancer cells will bind to newly synthesized cyclin E and block its folding/assembly, transport to the nucleus and interaction with CDK2, leading to inactivate cyclin E which may be closely related to tumor proliferation and transformation. **The goal of this proposal** is to define the role of altered cyclin E expression in breast tumorigenesis and cell proliferation, and to demonstrate whether cyclin E can be used as a therapeutic target for breast cancer therapy by the intrabodies or other approaches. Toward the goal of this study, in our preliminary study anti-cyclin E monoclonal hybridoma cell lines were generated, and the variable genes of these antibodies have been cloned and assembled into single-chain antibodies (sFvs). In this proposal, we will characterize the binding activity of the anti-cyclin E sFvs by using a recombinant phage antibody system and express the anti-cyclin E intrabodies targeted at the cytosol or nucleus of breast cancer cells. We will then generate breast cancer cell lines expressing the anti-cyclin E intrabodies and examine the effects on the cyclin E nuclear trafficking and associated kinase activity. Furthermore, we will evaluate the biological effects of the anti-cyclin E intrabodies on breast cancer cells and examine whether the anchorage-independent growth and tumorigenicity of breast cancer cells can be reversed by the anti-cyclin E intrabodies. This study should provide direct evidence to determine the functional significance of the cyclin E in the abnormal growth and transformation of breast cancer, and to demonstrate whether cyclin E can be used as a therapeutic target for breast cancer therapy by the intrabodies or other approaches.

Body

1. Experimental Outline

The present proposed study will be undertaken to obtain direct evidence whether cyclin E plays a critical role in establishing and maintaining the transformed phenotype of the breast cancer cells. To this end, we will express an anti-cyclin E intrabody at the cytosol or nucleus of breast cancer cells to generate phenotypic cyclin E "knock-out" cells. We will then determine the effects of intrabodies on the activities of cyclin E-associated protein kinase, and the cell proliferation and tumorigenicity of breast tumor cells. This proposed study of using the novel intrabody approach to inactivate cyclin E is also feasible to carry out in the applicant's laboratory. First, we have established the techniques of cloning and intracellular expression of engineered antibodies to inactivate a target protein. Second, the cellular location and structure of cyclin E has been defined. Cyclin E is a nuclear protein which is synthesized at the cytosol and then transported to the nucleus, where it associates with CDK2. The cyclin family shares a region of homology, called cyclin box, with all the cyclins isolated to date. This region is required on the cyclin E interaction with CDK2. Therefore, intrabodies against the cyclin box of cyclin E can inactivate the cyclin E function by blocking the interactions of with CDK2 as well as by inhibiting its nuclear trafficking. Third, anti-cyclin E hybridoma cell lines were generated and the variable region genes of the immunoglobulin heavy and light chains have been cloned for this proposed study. In summary, **the hypothesis of this proposed study** is that anti-cyclin E intrabodies targeted to the cytosol or nucleus of breast cancer cells will bind to newly synthesized cyclin E and block its folding/assembly, transport to the nucleus and interaction with CDK2, leading to inactivate the cyclin E functions which may be closely related to breast cancer transformation and cell proliferation. **The goal of this study** is to define the role of cyclin E in breast tumorigenesis and cell proliferation, and to demonstrate whether cyclin E can be used as a therapeutic target for breast cancer therapy by the intrabodies or other approaches.

2. Results and Discussion

I. Cloning and sequencing of variable region genes of anti-cyclin E intrabodies.

The rearranged V_H and V_L cDNA genes from mRNA of two anti-cyclin E hybridomas were PCR-amplified and sequenced. The cDNA sequences after comparing with the sequences in Genebank are unique. The heavy chain and light chain variable region cDNA sequences are as follows:

Anti-cyclin E (HE 12) Hybridoma

V_H

5'-GGCCGGAGGA GGCTTAGTGC AGCCTGGAGG GTCCCGGAAA CTCTCCTGTG
CAGCCTCTGG ATTCACTTTC AGTAGCTTTG GAATGCACTG GGTTCGTCAG
GCTCCAGAGA AGGGGCTGGA GTGGGTCGCA TACATTAGTA GTGGCAGTAG
TACCCTCCAC TATGCAGACA CAGTGAAGGG CCGATTCACC ATCTCCAGAG
ACAATCCCAA GAACACCCTG TTCCTGCAAA TGAAACTACC CTCACTATGC
TATGGACTAC TGGGGTCAAG GAACCTCAGT CACCGTCTCC TCATCTAGAA
AA-3'

V_K
5'-ACTCACTTTG TCCATGACAT GGAACAGTCA GCCTCCATCT CTTGCAAGTC
AAGTCAGAGC CTCTTAGATA GTGATGGCGA GACATATTTG AATTGGTTGT
TACAGAGGCC AGGCCAGTCT CCAAAGCGCC TAATCTATCT GGTGTCTAAG
CTGGACTCTG GAGTCCCTGA CAGGTTCACT GGCAGTGGAT CAGGGACAGA
TTTTACACTG AAAATCAGCA GAGTGGAGGC TGAGGATTTG GGAGTTTATT
ATTGCTGGCA AGTTACACAT TTTCCTCAGA CGTTCGGTGG AGGCACCAAG
CTGGGGATCA AACGTGCGGC GGCTTTAA-3'

Anti-cyclin E (HE 172) Hybridoma

V_H
5'-GGTCGGGCAG GATGGTGAAC CTTCGGAGAC CTGTCCCTCA CCTGCACTGT
CTCTGGAGGC TCCATCAGTA GTCACTACTG GAGCTGGATC CGGCAGTCCC
CAGGGAAGGG ACTGCAGTGG ATTGGATATA TCTACTACAG TGGGAGCACC
AACTACAGCC CCTCCCTCAA GAGTCGAGTC ACCATATCAG TAGAGACGTC
CAAGAACCAG TTCTCCCTGA AGCCGACCTC TATGACCGCT GCGGACACGG
CCGTGTGTTA CTGTGCGCGA AGCCCCGTGC CAGCTGTCTT CTACGGTGAC
TACCGACTCG ACCCCTGGGG GCAGGGAACC CTGGTCACGG TCACCTCAAA
AGG-3'

V_K
5'-AGGGGCTGGT TGCTTCCTTA GCTGTATCTC TGGGGCAGAG GGCCACCATC
TCATACAGGG CCAGCAAAAG TGTCAGTACA TCTGGCTATA GTTATATGGA
CTGGAACCAA CAGAAACCAG GACAGCCACC CAGACTCCTC ATCTATCTTG
TATCCAACCT AGAATCTGGG GTCCCTGCCA GGTTCAGTGG CAGTGGGTCT
GGGACAGACT TCACCCTCAA CATCCATCCT GTGGAGGAGG AGGATGCTGC
AACCTATTAC TGTCAGCACA TTAGGGAGCT TTCCTACACG TTCGGAGGGG
GGACCAAGCT GGAATCA-3'

II. Construction of various expression vectors

The heavy chain and light chain variable region cDNA sequences were assembled into sFvs in our preliminary study. The functional characterization of anti-cyclin E sFvs will be performed by using the recombinant phage antibody system (Pharmacia). For selection of sFvs with binding activity, the PCR-amplified sFv fragments will be cloned into the phagemid pCANTAB, which contains a minor coat protein at the tip of the phage, the gene 3 protein (g3p). To detect binding activities, we will take the advantage of the design that sFv can be produced either for phage display (by growth in supE strains of E.coli) or as a tagged soluble fragment (in non-suppresser strains such as HB2151) as the vector has an amber codon between the antibody and g3p. To do so, phagemids will be grown in E.coli HB2151 and induced with IPTG to allow production of soluble sFvs. The binding activity of sFvs will be detected by ELISA and Western-blotting. After selection, the pCANTAB 5E vectors containing cyclin E-binding sFvs will be sequenced by Sanger's method. This phage antibody system has been used in our laboratory to successfully select anti-HIV sFvs (unpublished data).

These results achieve the technically most challenged part of this proposed study--Technical Objective 1: cloning and charactirzation of genetically engineered anti-cyclin E antibodies. These results also lay a solid foundation to achieve the Technical Objectives 2: Characterization of anti-cyclin E sFvs and expression of intrabodies in mammalian cells, and Technical Objectives 3: Analysis of effects of intrabodies on breast cancer cells.

3. Experimental Methods

I. Effects on cyclin E nuclear trafficking and associated kinase activities

The anti-cyclin E sFvs with specific binding activities will be used to express intracellularly to inactivate cyclin E functions. In our previous study, sFv intrabodies can be stably expressed and targeted at the cytosol and nuclear compartments of eukaryotic cells, and the newly synthesized sFv intrabodies can fold to form a functional antigen-binding site at the reducing environment of the cytosol. The nuclear targeting of the anti-Tat sFv was not required to inhibit the function of Tat, a nuclear protein, because the anti-Tat intrabodies at the cytosol were able to bind newly synthesized Tat at the cytosol and block its nuclear transport. This result indicates that inactivation of a nuclear protein can be achieved by blocking the protein nuclear trafficking. Accordingly, anti-cyclin E sFv genes with or without a nuclear localization signal will be cloned into a mammalian expression vector pRc/CMV (Invitrogen) under the control of cytomegalovirus (CMV) promoter, which have been used to produce a high level of sFvs in mammalian cells in our previous studies (1-5). These anti-cyclin E intrabody expression vectors will be identified by restriction enzyme digestion and confirmed by DNA sequence analysis.

We will then examine whether the anti-cyclin E intrabodies can be stably expressed and targeted at the cytosol or nucleus of mammalian cells. To examine the expression and stability of anti-cyclin E intrabodies in mammalian cell, COS cells will be transfected with the vector DNA using lipofectin (Gibco-BRL), pulse-radiolabeled with ^{35}S-cysteine (ICN), and then chased for various times (6). The cells will then be lyzed and immunoprecipitated with a mixture of anti-mouse IgG antibodies (Sigma, Jackson Lab.). The stability of anti-cyclin E and its derivatives will be determined by a Phosphoimager (Molecular Dynamic). In addition, the localization of the expressed anti-cyclin E intrabodies in cells will be examined by immunofluorescent staining as described previously. The immunofluorescent staining pattern of anti-cyclin E intrabodies and their derivatives at the cytosol or nucleus will be determined under fluorescent microscopy. As we experienced previously, some sFv intrabodies were quite unstable in the cytosol for undefined reasons. In case that happens, additional modified expression vectors will be constructed, such as tagged with a human C_L sequence, since that the addition of the C_L domain was found to increase stability of the sFv intrabody. The expression of the modified intrabodies in mammalian cells will then be evaluated. If anti-cyclin E intrabodies or their derivatives are stably expressed, whether the anti-cyclin E intrabodies can block its nuclear transport and interaction with CDK2 will then be evaluated.

Breast cancer cell lines, such as MCF-7 or SKBR3 (available in our lab) which express a high level of cyclin E proteins and induce tumors in nude mouse will be used to evaluate the effects of anti-cyclin E intrabodies. First, we will evaluate whether the anti-cyclin E intrabodies or their derivatives are able to bind cyclin E in vivo by co-immunoprecipitation assays. To do so, the tumor cells grown on culture dishes will be transfected with the expression vector DNAs, and 48 hr later radiolabeled as described. The cell lysates will be immunoprecipitated with a polyclonal anti-cyclin E antibody (Santa Cruz Biotech, Inc). The immunoprecipitated samples will be analyzed by SDS-PAGE and visualized by a Phosphoimager. It is anticipated that cyclin E will co-precipitate the anti-cyclin E intrabodies, and inhibit the association of cyclin E with CDK2, if the anti-cyclin E intrabodies are able to bind the cyclin box domain of cyclin E. Second, we will determine whether the nuclear transport of newly synthesized cyclin E will be inhibited in the cells expressing the intrabodies at the cytosol by immunofluorescent staining. To do so, MCF or SKBR3 cells

grown on coverslips will be transfected with or without the anti-cyclin E vector DNAs, and 48 hr later, the cells will be fixed and stained with the polyclonal anti-cyclin E (1). The nuclear staining patterns of cyclin E observed in the untransfected tumor cells will be compared with that in the tumor cells expressing anti-cyclin E intrabodies. Tumor cells will also be transfected with a plasmid expressing irrelevant anti-Tat intrabodies as a negative control. If nuclear staining patterns of cyclin E in the cells expressing the anti-cyclin E intrabodies are altered, we will then evaluate their biologic effects on breast cancer cells.

We will first determine the role of cyclin E in cells by generating human fibroblast cells such as KD and IMR90 stably expressing anti-cyclin E intrabodies. Then, transformed breast cancer cell lines (MCF-7 and SKBR3) expressing the anti-cyclin E intrabodies will be established. A breast cancer cell line or human fibroblast line expressing an irrelevant anti-HIV-tat intrabody will also be generated for negative control. If the transformed cell lines are unable to establish because the expression of the anti-cyclin E antibodies disrupt the cyclin E vital functions for cell survival, inducible promoters, such as tetracycline or metallothione inducible promoter (available in the P.I.'s lab) will be used. After the cell lines constitutively or inducibly expressing anti-cyclin E are generated, we will then test whether the expression of anti-cyclin E intrabodies can lead to a reduced cyclin E-associated kinase activity. Assays for cyclin E associated kinase activity will be performed by measuring the phosphorylation of histone H1 in immunoprecipitates made with the anti-human cyclin E as described. In brief, exponentially growing cells will be lysed and these cell lysates will be incubated with the polyclonal anti-cyclin E or with nonimmune serum control and then incubated with protein A-Sephorose beads. The beads will be collected by centrifugation and washed. Kinase activities will be evaluated by using histone H1 proteins as substrates. The products of the reactions will be analyzed on a SDS-PAGE gel, and the radioactivity of the histone H1 band will be measured by a Phosphoimager.

II. Effects of anti-cyclin E intrabodies on breast cancer cells

To determine the biological effects of anti-cyclin E intrabodies, several biological aspects of breast cancer cells will be evaluated. First, we will determine the doubling times and saturation density of fibroblast or tumor cells with or without expression of anti-cyclin E intrabodies. To do so, the cells expressing anti-cyclin E or anti-Tat (control) will be plated in triplicate at a density of 1×10^4 per well in six well plates in 3 ml of RPMI plus 10% FCS. The number of cells per well will be counted using a Coulter counter every 2 days for the subsequent 14 days, and the doubling time and saturation density of each cell line will be calculated. Second, we will determine the cloning efficiency of the transduced or untransduced cells as follows: cells will be seeded in triplicate with 1,000 cells per 10 cm dish in a culture medium and will be refed with fresh medium every other day. The cells will be fixed and stained with 5% Giemsa after 10 days of growth and the number of grossly visible colonies will be counted. Third, we will evaluate the cell cycle distribution of the transduced or untransduced cells by flow cytometric analysis. Briefly, cells will be plated at 7×10^5/10 cm dish and incubated for several days. When the cells are exponentially dividing, the cells will be collected and analyzed for the cell cycle distribution and cell size by flow cytometry as described.

Anchorage-independent growth is the phenotypic property most tightly linked with tumorigenic behavior in vivo. We will examine whether these properties will be altered in the cancer cells expressing the cyclin E intrabodies. A HUT12 cell line that is an anchorage-independent, cyclin E-expressing transformed human fibroblast line and human breast cancer cell lines expressing anti-cyclin E intrabodies will be used. Transformed HUT12 cells expressing anti-cyclin E or anti-Tat (control) will

be generated. We will examine the formation efficiency of colonies in soft agar of the transformed cells to determine the anchorage-independent growth capability. Assays for growth in 0.3% Noble agar (Difco) will be performed as described. In brief, 8×10^4 cells will be suspended in RPMI plus 20% FCS containing 0.3% agar and plated in triplicate in six well plates. After 3 weeks of growth, the cells will be stained and the colonies will be counted under microscopy. In case that cell lines constitutively expressing intrabodies are unable to form colonies, cell lines inducibly expressing intrabodies will be used, and induced at different times to observe the effects of intrabodies. Finally, we will test the breast cancer cells expressing intrabodies for tumorigenicity in athymic (nude) mice following subcutaneous injection. To perform the tumorigenicity assays, 5×10^6 cells transformed breast cancer cells will be injected subcutaneously into multiple sites of athymic (nude) mice. The animals will be monitored for tumor formation every week and sacrificed 2 months later. The difference of tumor formation of the breast cancer cells expressing anti-cyclin E or anti-Tat (control) intrabodies will be determined.

Conclusions

From the study of last ten months, significant progress has been made. The major results are summarized as follows:

1. Cloning and sequencing two anti-cyclin E hybridoma heavy chain and light chain variable region cDNA.

2. Analyzing the two anti-cyclin E hybridoma heavy chain and light chain variable region cDNA by comparing with the sequences in the Genebank. These heavy chain and light chain variable region cDNA are unique.

3. Construct various vectors for expression of the anti-cyclin E in a single chain antibody form.

These results lay the foundation for further evaluation of cyclin E role in breast cancer tumorigenesis. The goal of this proposal to determine the cyclin E role in breast cancer tumorigenesis can be accomplished in next one or two years.

References

1. Marasco, W.A., Haseltine, W.A., and Chen, S.Y. Design, intracellular expression, and activity of a human anti-HIV-1 gp120 single chain antibody. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7889-7893 (1993).

2. Chen, S.Y., Bagley, J. and Marasco, W.A. Intracellular antibodies as a new class of molecules for gene therapy. *Human Gene Therapy*. **5**, 595-601 (1994).

3. Chen, S.-Y. Yousef, K. Bagley, J. Sodroski, J. and W.A. Marasco. Combined intra- and extracellular immunization against HIV-1 infection using a human anti-gp120 antibody. *Proc. Natl. Acad. Sci. USA.* **91**, 5932-5936 (1994).

4. Biocca, S., Neuberger, M.S., and Cattaneo, A. Expression and targeting of intracellular antibodies in mammalian cells. *EMBO J* **9**, 101-108. (1990)

5. Werge, T.M., Biocca, S. and Cattaneo, A. Intracellular immunization: Cloning and intracellular expression of a monoclonal antibody to the p21ras protein. *FEBS Lett.* **274**, 193-198. (1990)

6. Mhashikar, A.M., Bagley, J., Chen, S.-Y., Szilvay, A.M. Helland, D.G. and Marasco, W. Inhibition of HIV-1 infection using cytoplasmic anti-Tat antibodies. *EMBO J.* **14**, 1542-1551 (1995).

Final Report

The results from this study are not published yet.
An abstract was submitted to Cold Spring Harbor Gene Therapy Meeting (Sept. 23-27, 1998).

No personnel receive any pay (not salaries) from this effort